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Inventor(s): BERND BACHMANN, GEORG THIERBACH, JORN KALINOWSKI and ALFRED PUHLER

Invention: PROCESS FOR THE FERMENTATIVE PRODUCTION OF L-LYSINE

Cushman, Darby & Cushman
1615 L Street, N.W.
Eleventh Floor
Washington, D.C. 20036-5601
Attorneys
Telephone: 861-3000

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SPECIFICATION

METHOD FOR THE FERMENTATIVE PRODUCTION OF L-LYSINE

The pres nt invention relates to a method for the fermentative production of L-lysine.

Corynebacterium glutamicum and related genera, such as Brevibacterium lactofermentum and Brevibacterium flavum, are known as microorganisms which form amino acids. In order to increase productivity, artificial mutations have been carried out. Examples of mutants produced in this manner include lysine-producing strains of C. glutamicum which exhibit, in addition to an AEC-resistance (AEC = S-2-aminoethyl cysteine), a homoserine and leucine auxotrophy coupled thereto (US 3,708,395), or sensitivity to methionine (US 3,871,960).

In addition to these classic methods, vector systems have been developed which make possible the transformation of microorganisms of the genera Corynebacterium and Brevibacterium (DE-OS 37 37 719, DE-OS 38 41 453, G. Thierbach, A. Schwarzer, A. Puhler, "Appl. Microbiol. Biotechnol" 29 (1988), pp. 356-362).

EP-A-0,219,027 describes a method for the production of various amino acids in which microorganisms of the genera Corynebacterium and Brevibacterium are transformed with recombinant DNA, thereby increasing the amount of amino acids excreted. The recombinant DNA contains a DNA fragment which codes for the synthesis of aspartate semialdehyde dehydrogenase or aspartate amino transferase.

US 4,346,170 teaches the cloning of genetic information into E. coli, which information controls the formation of lysine and is derived from a strain of the same genus having a resistance to a L-lysine analogue, such as AEC.

The subject matter of US 4,560,654 is similar; however, according to this patent, genetic information from an AEC-resistant strain of Corynebacterium is cloned into a lysine-auxotrophic strain of <u>C. glutamicum</u> with the result that lysine is excreted. The identity of the cloned DNA fragment is not disclosed.

It appears from EP-A-88,166 also that a strain of <u>C. glutamicum</u> excretes lysine after it has acquired the phenotype of AEC resistance by transformation. The recombinant plasmid pAec5 used for this purpose contains a 3.9 kb fragment of chromosomal DNA inserted at the BglII cleavage site of the vector pCG 11. The invention has the problem of regulating an enzyme important in lysine biosynthesis in a microorganism of the genus Corynebacterium or Brevibacterium in a manner such that either a lysine excreter results or the rate of lysine excretion is increased.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1: Restriction map of plasmid pZ1. The thick line represents the pHM1519 portion and the thin line represents the pACYC177 portion of pZ1;
Ap^R: ampicillin resistance gene; Km^R: kanamycin resistance gene.

Figure 2: Restriction map of plasmid pCS2 in linearized form. The upper part of the Figure shows the positions of various restriction cleavage sites. The lower part of the Figure shows various regions of plasmid pCS2. The insertions of DM58-1 DNA are shown as an open bar. The ampicillin resistance gene of pZ1 is

emphasized in black and the kanamycin resistance gene is characterized by dots. The other pZ1 portions of pCS2 are emphasized by shading. Abbreviations: BamHI = B; BclI = C; SalI = S; Sca = A; SmaI = M; and XhoI = X.

Figure 3: Deletion map of plasmid pCS2. The upper part of the Figure shows the derivatives derived from pCS2 and the lower part shows the derivatives derived from pCS23. The ampicillin resistance gene of pZ1 is emphasized in black; the kanamycin resistance gene is shown in dots; the other pZ1 portions of pCS2 are characterized by shading. The insertion of DM 58-1 DNA is shown as an open bar. The deletions are characterized as a line. Abbreviations: BamHI = B; BclI = C; DraI = D; EcoRI = E; SalI = S; ScaI = A; SmaI = M; and XhoI = X.

Figure 4: Deletion analysis and sequencing strategy of the chromosal fragment of plasmid pCS2. Deletion analysis: The plasmids pCS23 and pCS24 impart, just as clone pCS2, AEC resistance and lysine production. The shaded bars represent the vector portion of the plasmids. Sequencing strategy: The 2.1 kb PstI-KhoI fragment of plasmid pCS24 was subcloned with the sketched-in restriction cleavage sites. The arrows indicate the sequenced range and the direction of reading. The restriction cleavage sites of the enzymes DraI (D), EcoRI (E), BglII (G), HindIII (H), NaeI (N), PstI (P), SalI (S) and XhoI (X) are

drawn in. The two open reading frames are shown thereunder which code for the subunits of the aspartate kinase and for aspartate-ß-semialdehyde dehydrogenase.

Figure 5: DNA sequence and derived amino acid sequence of the 2.1 kb PstI - XhoI fragment. The amino acid sequences of ORF 1 (1-794) and ORF 2 (821-1846) are indicated in the 3-letter code. The numbering below the DNA strand refers to the DNA sequence. · The names of the cloning cleavage sites used for sequencing are likewise entered underneath. Ribosome binding sites are marked by asterisks (*), start condons by arrows (->) and the terminator structure by bars (-).

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a method of producing L-lysine. According to the present method, there is inserted into an optionally lysine-producing microorganism of the genus Corynebacterium or Brevibacterium, recombinant DNA that includes: (i) a DNA fragment comprising a genetic sequence that codes for the production of a protein which results in an aspartyl-β-semialdehyde dehydrogenase (asd) activity and/or a protein that results in the deregulation of the aspartate kinase (lysC), which sequence is derivable from a microorganism of the genus Corynebacterium or Brevibacterium, and (ii) a vector. The transformant obtained in this manner is grown in a suitable, known medium and the L-lysine formed is separated therefrom using known methods.

Donor strains suitable for use in the present invention are, preferably, L-lysine-producing

bacteria of the genera Br vibacterium or Corynebacterium which contain the appropriate DNA sequenc s. C. glutamicum DM 58-1, developed by mutagenesis of Corynebacterium ATCC 13032 with ethylmethane sulfonate, and exhibiting AEC resistance, is particularly preferred. This strain is deposited under the accession number DSM 4697, and is host bacterium for plasmid pDM6. One skilled in the art can separate the latter according to known methods and obtain strain DM58-1 (FEMS Microbiology Review 32 (1986), pp. 149 - 157). Chromosomal DNA is extracted from the donor in a known manner and treated with restriction endonucleases. After constructing the recombinant DNA by introducing the chromosomal DNA fragment into a vector, the microorganism is transformed with the plasmid thus obtained, for example, with pCS2, the restriction map of which is shown in Figure 2. The plasmid pCS2 has been deposited in the strain C. glutamicum DM2-1/pCS2 under the accession number DSM 5086 in the German Collection for Microorganisms and Cell Cultures in accordance with the Budapest Agreement.

A preferred vector system is pZ1 (deposited in C. glutamicum DM 274-2 under accession number DMS 4241) or also pCV34, pCV36, pCVX4, pCVX10, pCVX15, pZ9 and pZ8-1 (DE-OS 38 41 454.6) or pCV35, pECM3, pECM1 (DE-OS 38 41 453.8). However, the plasmids known from EP-A-93 611, to the extent that they replicate in Corynebacteria or Brevibacteria, especially pAJ 655, pAJ 611, pAJ 440, pAJ 1844 and pAJ 3148, but also pCG 11, pCE 54 (cf. EP-A 0,233,581), and likewise pUL330 (R.I. Santamaria et al., "J. Bacteriology" 162 (1985), pp. 463-467), can be used as well.

The present invention also relates to microorganisms of the genera Corynebacterium or Brevibacterium containing th above-described

recombinant DNA, and to the use of such microorganisms in the production of L-lysine by fermentation.

The cloned DNA fragment (see Figure 2) contains only a fraction of the aspartat kinase gene (lysC) as well as the complete gene of the aspartyl-β-semialdehyde dehydrogenase (asd), as will be appreciated from analysis of the sequence. The fraction of the aspartate kinase gene comprises a DNA sequence homologous to the β subunit of the aspartate kinase II from <u>B. subtilis</u>.

All transformants whose plasmid exhibits this sequence (pCS2, pCS21, pCS22, pCS23, pCS24, pCS26, pCS233) contain an aspartate kinase which is clearly desensitized compared to the chromosomally coded enzyme from ATCC 13032 with respect to the feedback inhibitors L-lysine and L-threonine, and exhibit AEC resistance.

The conclusions draw from comparison of homology was that the PstI - XhoI gene fragment from DM58-1 harbors only a part of the lysC gene (AK), but the complete asd gene was able to be unambiguously confirmed by means of enzyme measurements. Surprisingly, no <u>C. glutamicum</u> ATCC 13032 strain transformed with pCS2 or a pCS2 derivative contains an aspartate kinase activity which is elevated in comparison to the recipient strain (Table 4, column 3). In contrast, a strong overexpression of the aspartyl-β-semialdehyde dehydrogenase (ASA-DH) can be demonstrated in all transformants whose plasmids contain the asd structure gene (Table 4, column 2, Figures 3 and 4). The plasmids pCS23 and pCS23 derivatives do not, as expected, result in an overexpression of ASA-DH.

The strong overexpression of ASA-DH, which occurs during the cloning of the DNA fragment of the invention with the aid of pCS 2 and derivatives derived therefrom, assures an efficient conversion

of.the product of the aspartate kinase reaction, β-aspartyl phosphat , as a result of which an acceleration of the no-long r inhibitable aspartate kinase reaction occurs. Due to the high lability of ASA-DH, the factor of overexpression which can be calculated from the specific activity varies from 31 to 65.

A considerable simplification over the state of the art results from the fact that, in the first place, only a fraction of the lysC gene, which results in a deregulation of the aspartate kinase, must be isolated in order to bring about, or improve, an excretion of lysine. In the second place, the organization of lysC and asd in one operon permits the asd gene to be isolated together with the lysC gene without additional expense, due to the AEC resistance occurring with the mutated lysC gene. Alternatively, the DNA fragment containing lysC and asd can be isolated with the aid of asd mutants independently of whether lysC is mutated or not.

EXAMPLES

1. Characterizing of gene donor DM58-1 and gene receiver ATCC 13032

1.1 Development and phenotype of strain DM58-1

Strain DM58-1 was developed by means of mutagenesis of *C. glutamicum* strain ATCC 13032 using a customary concentration of ethylmethane sulfonate.

The selection took place by plating out the mutant mixture obtained in this manner on minimum agar having the composition: 20 g glucose, 10 g $(NH_4)_2SO_4$, 2.5 g urea, 1 g KH_2PO_4, 0.4 g $MgSO_4 \cdot 7H_2O$, 2 mg $FeSO_4 \cdot 7H_2O$, 1.5 mg $MnSO_4 \cdot H_2O$, 300 μg biotin, 900 μg thiamine and 20 g agar per liter distilled water (pH 7.0), and containing a suitable concentration of

S-amino-ethyl-D,L-cysteine (AEC). A clone, later designated as DM58-1, which grows on this medium and is isolated from such a sel ction medium, carries no genetic markers other than its AEC resistance.

1.2 Levels of aspartate kinase and aspartyl-beta-semialdehyde dehydrogenase in ATCC 13032 and DM58-1

The strains ATCC 13032 and DM58-1 were cultivated under directly comparable conditions in standard I broth (Merck Art. Nr. 7882) with an additional 4 g/l glucose and 1 mM $MgCl_2$ at 30°C and 150 rpms until attainment of the early stationary phase, and then separated by means of centrifugation from the culture medium. The cells were washed 3 times with 100 mM Tris/HCl (pH 7.5); 1 mM DTT and the moist cell mass suspended in one volumetric part of the same buffer.

The cells suspended in this manner were disrupted in a ball mill (B. Braun Melsungen - MSK homogenizer, IMA disintegrator S) by means of agitation with a suitable amount of glass beads. The cell homogenate was separated from the glass beads by means of a glass-filter and clarified by centrifugation for 30 minutes at 30000 x g.

After 15 hours of dialysis in an enzyme-stabilizing buffer, the enzyme activities were determined in the following test mixtures:

Aspartate-kinase test: 100 mM Tris/HCl (pH 7.5), 1 mM DTT, 400 mM $(NH_4)_2SO_4$, 20 mM $MgCl_2$, 400 mM $NH_2OH \cdot HCl$, 300 mM L-aspartate, 40 mM ATP and various amounts of enzyme preparation.

The enzyme reaction is stopped after 30 minutes incubation at 37°C by means of the addition of 750 µl of a solution consisting of 10 % $FeCl_3 \cdot 6H_2O$; 3.3 % TCA, and 0.7 N HCl to 500 µl of the enzyme t st

mixture. The enzyme activity expressed in μmoles/mg·min (U/mg), is calculated from the aspartyl-beta-hydroxamate concentration determined photometrically ($\Delta E_{540\ nm}$) using a calibration curve. The associated protein concentrations were determined according to the method of Lowry et al. ("J. Biol. Chem." 193, p. 265, (1951)) or Bradford ("Anal. Biochem." 72, p. 248, (1976)).

The aspartyl-β-semialdehyde dehydrogenase test contains 120 mM diethanolamine (pH 9.0), 40 mM Na_2AsO_4, 1 mM $NADP^+$, 5 mM L-threonine, 1,3 mM aspartyl-beta-semialdehyde and various amounts of enzyme preparation in a total volume of 1 ml. The activity, indicated in μmoles/mg·min. (U/mg), is calculated from the rate of NADPH synthesis determined photometrically ($\Delta E_{540\ nm}$).

Table 1 contains the specific enzyme activities of both enzymes in raw extracts of identically grown and worked-up cells of C. glutamicum ATCC 13032 and DM58-1. In addition to comparable contents of aspartate kinase of both strains, the AEC resistant mutant contains DM58-1 aspartyl-β-semialdehyde dehydrogenase activity elevated approximately 5-fold in comparison to the wild type.

1.3 In vitro capacity of aspartate kinase from C. glutamicum ATCC 13032 and DM58-1 to be inhibited

Table 1 shows the reproducibility of the extent of inhibition of C. glutamicum wild type enzyme, previously indicated by K. Nakayama et al. ("Agr. Biol. Chem." 30, p. 611, (1966)) and more closely examined by S.N. Kara-Murza et al. ("Prikladnaya Biokhimiya: Microbiologia" 14, p. 345, (1978)). This is contrasted by the distinctly different character of the enzyme of the AEC resistant

mutant DM58-1, aspartate kinas which can no longer by inhibited in a concerted fashion by L-lysine and L-threonine. The enzyme of the mutants is likewise influenced only slightly by S-aminoethyl-D,L-cyst ine (AEC), acting similarly on the enzyme from ATCC 13032.

TABLE 1

Enzyme contents and properties of aspartate kinase (AK) and aspartyl-β-semialdehyde dehydrogenase (ASA-DH) from C. glutamicum ATCC 13032 and DM58-1

Strain	ATCC 13032	DM58-1
AK (U/mg)	0.016	0.011
ASA-DH (U/mg)	0.06	0.33
Inhibitor Combinations	AK-inhibition (%)	
10 mM L-Lys 1 mM	89	12
10 mM L-Lys 10 mM L-Thr	95	2
100 mM L-Lys 10 mM L-Thr	99	21
10 mM AEC 1 mM L-Thr	12	0
10 mM AEC 10 mM L-Thr	41	0
100 mM AEC 10 mM L-Thr	95	7

abbreviations: AEC S-(aminoethyl)-D,L-cysteine

2. Cloning of a DNA fragment of C. glutamicum strain DM58-1 coding for a feedback-resistant aspartate kinase

2.1 Cloning

Total DNA was isolated from C. glutamicum strain DM58-1, as described by Chater et al. ("Curr. Topics Microb. Immunol." 96, p. 69, (1882)), and partially digested with the restriction enzyme PstI. The vector pZ1 (Figure 1), which is described in German patent application 37 37 729.9, was linearized with PstI and dephosphorylated by treatment with alkaline phosphate. Vector DNA and DM58-1 DNA were mixed and treated with T4 DNA ligase as described by Maniatis et al. (Molecular Cloning, A Laboratory Manual, Cold Spring Harbour Laboratory, 1982).

The transformation of C. glutamicum ATCC 13032 with the ligation mixture took place as described by Thierbach et al. ("Applied Microbiology and Biotechnology" 29, p. 356, (1988)).

The transformation mixture was plated out onto RCG/E-agar with 300 µg/ml kanamycin and the agar plates incubated one week at 30°C. The agar plates were subsequently stamped over onto MM agar ("J. Bact." 159, p. 306 (1984)) with 50 mM AEC and 50 mM L-threonine and incubated one day at 30°C. A colony which was able to grow on this agar was plated out on MM agar containing AEC, L-threonine and 10 µg/ml kanamycin in order to obtain individual colonies. Plasmid DNA was isolated from such a clone, designated as pCS2 and used for the transformation of C. glutamicum ATCC 13032. Fifty nine of 62 kanamycin-resistant transformants tested proved to be resistant to inhibition by 50 mM AEC and 50 mM L-threonine. Plasmid pCS2 was characterized further by restriction mapping. It contains an insertion approximately 9.9

kb long in the PstI cleavage site of vector pZ1, which has a length of 5.9 kb. The restriction map of pCS2 is shown in Figure 2.

2.2 Characterization of the aspartate kinase activity

Aspartate kinase activity was measured in strain ATCC 13032/pCS2 as a positive check in strain DM58-1 and as a negative check in strain ATCC 13032. The strains were cultivated in standard I broth supplemented with 4 g/l glucose, 10 μg/ml kanamycin and 1 mM $MgCl_2$. Culture conditions, cell harvest, cell digestion and determination of the aspartate kinase were carried out as described in Section 1.2 above. The effectors L-Lys, L-Thr and AEC were added in each instance as stock solutions in 100 mM Tris/HCl buffer with a pH of 7.5.

Aspartate kinase content and the capacity of the enzyme from ATCC 13032/pCS2 to be inhibited are shown in Table 4. Although the strain exhibited no elevated specific activity, a distinct desensitization in relation to the cited inhibitors was demonstrated, the extent of which, however, does not attain the degree of the deregulation of the enzyme from the gene donor DM58-1 (partial deregulation).

2.3 Determination of L-lysine excretion

The ability to excrete lysine was determined in strain ATCC 13032/pCS2 and as negative check in strain ATCC 13032/pZ1. After the addition of 10 μg/ml kanamycin, the culture was carried out as described below. The results of the test are collated in Table 2.

TABLE 2

Excretion of L-lysine by various C. glutamicum strains

C. glutamicum strain	Concentration of excreted L-lysine · HCl (g/l)
ATCC 13032/pZ1	0.0
ATCC 13032/pCS2 (= DM 2-1/pCS2)	7.1

A 100 ml Erlenmeyer flask with baffle is filled with 10 ml of the following culture medium: 12 g/l ammonium sulfate, 240 g/l molasses, 60 ml/l soybean flour hydrolysate and 10 g/l $CaCO_3$. After seeding, the cultures are incubated 72 hours at 30°C and 300 rpms. The lysine determination took place in the centrifuged supernatant with the aid of amino acid analyzers.

3. Deletion mapping of the DNA fragment of pCS2 which codes for a feedback-resistant aspartate kinase

Various deletion derivatives were constructed by means of the complete or partial digestion of pCS2 with various restriction enzymes and subsequent treatment with T4 DNA ligase at a low DNA concentration. The production of the various deletion derivatives is collated in Table 3, and the position of the deletions in the various derivatives is shown in Figure 3. The resistance behavior of the strains derived from C. glutamicum ATCC 13032 vis-à-vis AEC is likewise shown in Figure 3. In this manner, the DNA region imparting AEC resistance was able to be limited to a DNA fragment approximately 1.5 kb long which is

defined in plasmid pCS233 by the PstI cloning cleavage site and an EcoRI cleavage site.

TABLE 3

Production and $AEC^{R/S}$ - phenotype of various deletion derivatives of plasmid pCS2

Plasmid	Construction	$AEC^{R/S}$-phenotype
pCS21	produced after digestion of pCS2 with BamHI	R
pCS22	produced after digestion of pCS2 with BamHI and BclI	R
pCS23	produced after partial digestion of pCS2 with SalI	R
pCS24	produced after partial digestion of pCS2 with XhoI	R
pCS26	produced after digestion of pCS2 with ScaI	R
pCS231	produced after partial digestion of pCS23 with PstI	S
pCS232	produced after partial digestion of pCS23 with DraI	S
pCS233	produced after partial digestion of pCS23 with EcoRI	R

Abbreviations: R = resistance
S = sensitivity

The aspartate kinase activity and the capacity of the enzymatic activity to be inhibited by mixtures of lysine and AEC and threonine was determined in the constructed clones. Growth, digestion and determination of activity were carried out as described above. In addition, the ability of the various clones to excrete L-lysine was investigated. An agar plate diffusion test with an L-lysine-auxotrophic indicator strain from C. glutamicum was used to this nd. It can be seen from Table 4

that all AEC-resistant strains exhibit a partially deregulated aspartate kinase activity and are capable of xcreting L-lysine.

TABLE 4

Microbiological and biochemical characterization of recombinant *C. glutamicum* strains

Strain	ASA-DH (U/mg)	AK (U/mg)	AK residual activity (%) in the presence of 10mM Lys 1mM Thr	10mM Lys 10mM Thr	100mM Lys 10mM Thr	100mM AEC 10mM Thr	AEC^R	lysine excretion
ATCC13032 (pZ1)	0.06	0.016	9	5	3	7	-	-
ATCC13032 (pCS2)	3.9	0.013	55	55	28	56	+	+
ATCC13032 (pCS21)	n.b.	0.016	46	50	24	n.b.	+	+
ATCC13032 (pCS22)	n.b.	0.015	40	41	22	n.b.	+	+
ATCC13032 (pCS23)	0.03	0.014	51	57	30	56	+	+
ATCC13032 (pCS24)	2.07	0.011	65	65	40	62	+	+
ATCC13032 (pCS26)	1.88	0.015	64	63	39	60	+	+
ATCC13032 (pCS231)	0.060	0.013	11	14	4	10	-	-
ATCC13032 (pCS232)	n.b.	n.b.	n.b.	n.b	n.b.	n.b.	-	n.b.
ATCC13032 (pCS233)	n.b.	0.011	56	62	36	57	+	n.b.
DM58-1 (pZ1)	0.330	0.009	83	100	79	93	+	+

abbreviations: ASA-DH: aspartyl-β-semialdehyde dehydrogenase
AK: aspartate kinase
n.b.: not determined

4. Sequ ncing of a DNA fragment of plasmid pCS24 imparting the phenotyp AEC resistanc

4.1 <u>Sequencing m thod</u>

The nucleotide sequence of the 2.1 kb PstI-XhoI DNA fragment was determined according to the method of Maxam and Gilbert (A.H. Maxam et al., "Proc. Natl. Acad. Sci. USA" <u>74</u>, pp. 560 - 564, (1977)) with the modifications of Arnold and Puhler (W. Arnold et al., "Gene" 70, p. 171 ff, (1988)). The subcloning for the sequencing started from plasmid pCS24 (Figure 4). Plasmids pCS24 was transferred into the *E. coli* strain MM 294 (M. Merelson et al., "Nature" <u>217</u>, pp. 1110 - 1114, (1968)) and appropriate fragments cloned into the sequencing vectors pSVB21, 25 and 26 (W. Arnold et al., "Gene", 70, p. 171 ff, (1988)). Insertion inactivation was demonstrated in the *E. coli* strain JM83 (J. Messing, Recombinant DNA Technical Bulletin, NIH publication No. 79-99 <u>2</u>, pp. 43 - 48, (1979)) by means of the XGal test (5-bromo-4-chloro-indolyl-β-D-galactopyranoside).

The sequencing strategy is shown in Figure 4. The nucleotide sequence was determined from both DNA strands with overlapping clones.

4.2 <u>DNA sequence of the 2.1 kb PstI-XhoI-DNA fragment</u>

The sequenced DNA piece is 2112 bp long. It carries restriction cleavage sites for the enzymes BglII, DraI, EcoRI, HindIII, NaeI, PstI, SalI and XhoI, with which the subclones were also produced (Figure 5). The nucleotide sequence was processed with the sequence analysis program packet ANALYSEQ (R. Staden et al., "Nucl. Acids Res." <u>14</u>, pp. 217 - 232, (1986)).

Two long, open reading frames (ORF) are located on the sequenced DNA piece. Both ar located from the PstI cleavage sit to the XhoI cleavage site. Only a small range of 26 bp is located between the two. A ribosome binding site (RBS) (806-809 AGGA followed by the start codon ATG) is located in front of the 2nd ORF. An RBS (AGGA, 268-271 with start codon GTG) was likewise localized within the 1st ORF.

ORF 1 has a length of 264 amino acids (AA), calculated from the PstI site and 172 AA (corresponding to 18.6 k Dals) from the internal RBS. ORF 2 is 342 AA (36.1 Dals) long.

A possible transcription termination structure, a so-called hairpin loop, followed by several thymine groups (1864-1900) is located directly behind ORF 2. This arrangement is characteristic for ρ independent termination signals in E. coli and other bacteria species (Ahyda et al., "Ann. Rev. Biochem." 47, pp. 967 - 996, (1978)). The terminator present here exhibits a stability of more than -40 kcal/mole at 30°C.

A possible promoter for ORF 2 was determined within ORF 1 (490-437), TTGACA-17 bp-TATTCT). The -35 region and the distance to the -10 region correspond exactly to the E. coli consensus promoter (D.K. Hawley et al., "Nucl. Acids Res." 11, pp. 2237 - 2255, (1983)); the -10 region is very similar to the E. coli consensus region (TATAAT).

4.3 Analysis of the amino acid sequence

The amino acid sequences translated from ORF 1 and ORF 2 were compared with the known sequences of the aspartate kinases (AK) I (M. Cassan et al., "J. Biol. Chem." 261, pp. 1052 - 1057, (1986)) of E. coli and the AK II of B. subtilis (N.Y. Chen et al., "J. Biol. Chem." 262, pp. 8737 - 2255, (1987)) and the AA

sequences of the aspartate semialdehyde dehydrogenases (ASA-DH) of E. coli (C. Haziza et al., "Embo J." 1, pp. 379 - 384, (1982)) and Streptococcus mutans (G.A. Cardineau et al., "J. Biol. Chem." 262, 7, pp. 3344-3353, (1987)). The programs MALIGN (E. Sobel et al., "Nucl. Acids Res." 14, pp. 363 - 374, (1986)) and DIAGON (R. Staden et al., "Nucl. Acid Res." 14, pp. 217 - 232, (1986)) were used for this. Significant agreement between ORF 1 and the AK sequences on the one hand and ORF 2 and the ASA-DH sequence of S. mutans on the other hand became apparent. Only weak homologies to the E. coli ASA-DH became apparent, mainly, however, in the area of the active center (C. Haziza et al, "EMBO J." 1, pp. 379 - 384, (1982)).

The following results were obtained from the computer analyses:

- ORF 1 corresponds to the C terminus of the aspartate kinase, i.e., approximately 160 AA are lacking from the N terminus as well as the complete promoter region.

- ORF 2 corresponds to the aspartate semialdehyde dehydrogenase.

The homology of ORF 1 with the B. subtilis AK II is evident to an expert. The AK II consists of overlapping subunits (N.-Y. Chen et al., "J. Biol. Chem." 262, pp. 8787 - 8798, (1987)). The β subunit corresponds to the C terminus of the α subunit. Since the RBS found in ORF 1 coincides in its position exactly with the RBS of this AK, a conclusion by analogy can be made.

5. Expression experiments

5.1 Complementation of asd negative and lysC negative strains of E. coli

The identity of ORF 2 with the asd gene was proven by means of complementation of the asd-negative E. coli strain RASA 6 (F. Richaud et al., C.R. Acad. Sc. Paris 293, pp. 507 - 512, (1981)) by the plasmids pCS2 and pCS24. pCS23, in which approximately 50 amino acids are lacking from the C terminus of the ASA-DH, does not complement. None of these plasmids was capable of complementing the AKI-III negative E. coli Gif 106 M1 (E. Boy et al., "Biochemie" 61, pp. 1151 - 1160, (1979)).

5.2 Determination of the specific aspartate kinase (AK) and aspartyl-β-semialdehyde dehydrogenase (ASA-DH) in transformants of ATCC 13032 with various pCS2 deletion derivatives

The conclusions deduced by analogy in 4.3 from homology comparisons, according to which the PstI-XhoI gene fragment from DM58-1 harbors only a part of the lysC gene (AK), but the complete asd gene, were able to be unambiguously corroborated by means of enzyme measurements.

No C. glutamicum ATCC 13032 strain transformed with pCS2 or a pCS2 derivative contains an aspartate kinase activity which is elevated in relation to the recipient strain (Table 4, column 3).

In contrast, a strong overexpression of the ASA-DH was demonstrated in all transformants whose plasmids contained the asd structure gene (Table 4, column 2, Figures 3 and 4). The plasmids pCS23 and pCS23 derivatives did not, as expected, result in an overexpression of ASA-DH. Due to the high lability of

ASA-DH, the factor of overexpression which can be calculated from the specific activity varies from 31 to 65.

6. Enzyme properties and L-lysine excretion

The L-lysine excretion of 7.1 g/l in 72 hours demonstrated for ATCC 13032 pCS2 (Table 2) can be traced to two changes realized by means of genetic engineering:

a) Cloning of the regulation subunit of the aspartate kinase from DM58-1 without increase of the cellular enzyme content; and

b) cloning of the aspartyl-β-semialdehyde dehydrogenase from DM58-1, which results in a 31-65-fold increase of the cellular enzyme content.

WHAT IS CLAIMED IS:

1. A method of producing L-lysine comprising:

(1) transforming a microorganism of the genus Corynebacterium or Brevibacterium with a recombinant DNA molecule comprising:

(i) a DNA fragment comprising a genetic sequence which codes for the production of proteins which result in an aspartyl-β-semialdehyde dehydrogenase (asd) activity or in deregulation of the aspartate kinase (lysC), which sequence is derivable from a microorganism of the genus Corynebacterium or Brevibacterium, and

(ii) a vector;

(2) culturing said transformant under conditions such that L-lysine is produced; and

(3) isolating the L-lysine thus formed.

2. The method according to claim 1, wherein the recombinant DNA molecule is a plasmid that can replicate in said microorganism of the genus Corynebacterium or Brevibacterium.

3. The method according to claim 2, wherein the vector is selected from the group consisting of pZ1, pCV34, pCV36, pCVX4, pCVX10, pCVX15, pZ9, pZ8-1, pCV35, pECM1, and pECM3.

4. The method according to claim 1, wherein the recombinant DNA molecule is the plasmid pCS2 present in *Corynebacterium glutamicum* DSM 5086.

5. The method according to claim 4, wherein the recombinant DNA molecule is pCS21, pCS22, pCS24, or pCS26.

6. The method according to claim 4, wherein the recombinant DNA molecule is pCS23 or pCS233.

7. A microorganism of the genus Corynebacterium or Brevibacterium, comprising a recombinant DNA molecule comprising:

a DNA fragment comprising a genetic sequence which codes for the production of proteins which result in an aspartyl-β-semialdehyde dehydrogenase (asd) activity or in deregulation of aspartate kinase (lysC), and which sequence is derivable from a microorganism of the genus Corynebacterium or Brevibacterium, and

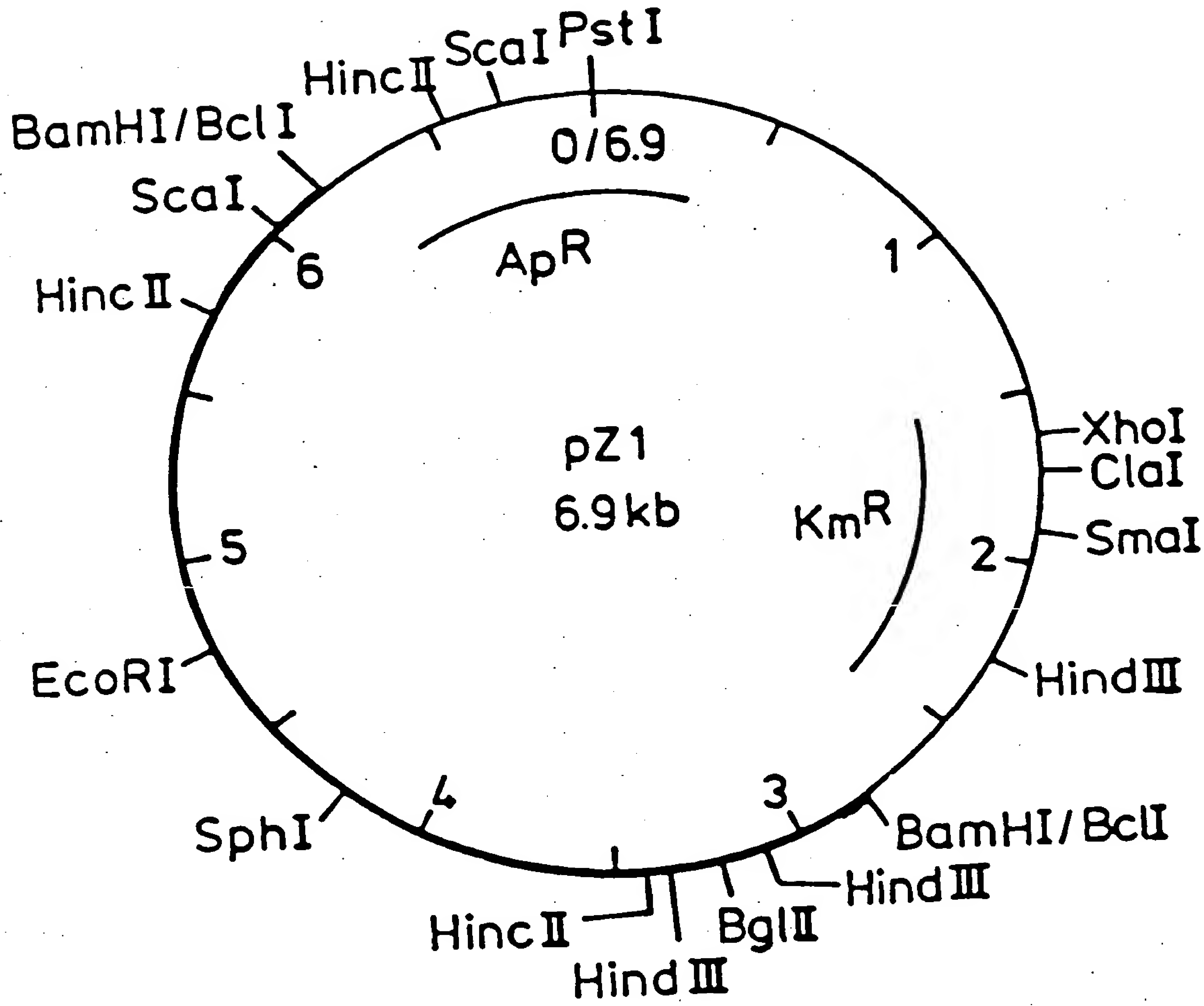
a vector.

8. A DNA fragment having a genetic sequence that codes for the production of proteins which result in an aspartyl-β-semialdehyde dehydrogenase (asd) activity or in deregulation of aspartate kinase (lysC) in a microorganism of the genus Corynebacterium or Brevibacterium with a length of 9.9 kb, as shown in Figure 2.

9. The DNA fragment according to claim 8, consisting essentially of a 2.1 kb genetic sequence defined by a PstI cleavage site and a XhoI cleavage site, encoding the amino acid sequence presented in Figure 5.

ABSTRACT OF THE DISCLOSURE

A method of producing L-lysine, in which recombinant DNA which consists of a DNA fragment comprising a genetic sequence which codes for the production of proteins which result in an aspartyl-β-semialdehyde dehydrogenase (asd) activity and/or in the deregulation of the aspartate kinase (lysC) and which sequence is derivable from a microorganism of the genus Corynebacterium or Brevibacterium, and a vector, is inserted into a microorganism of the genus Corynebacterium or Brevibacterium, the transformant obtained in this manner being grown in a suitable medium and the L-lysine thus formed being isolated therefrom.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ScaI
HincII
BamHI/BclI
ScaI
HincII
0/6.9
ApR
1
6
XhoI
ClaI
SmaI
2
pZ1
6.9 kb
KmR
5
EcoRI
HindIII
3
4
SphI
BamHI/BclI
HindIII
BglII
HincII
HindIII

Figure 1

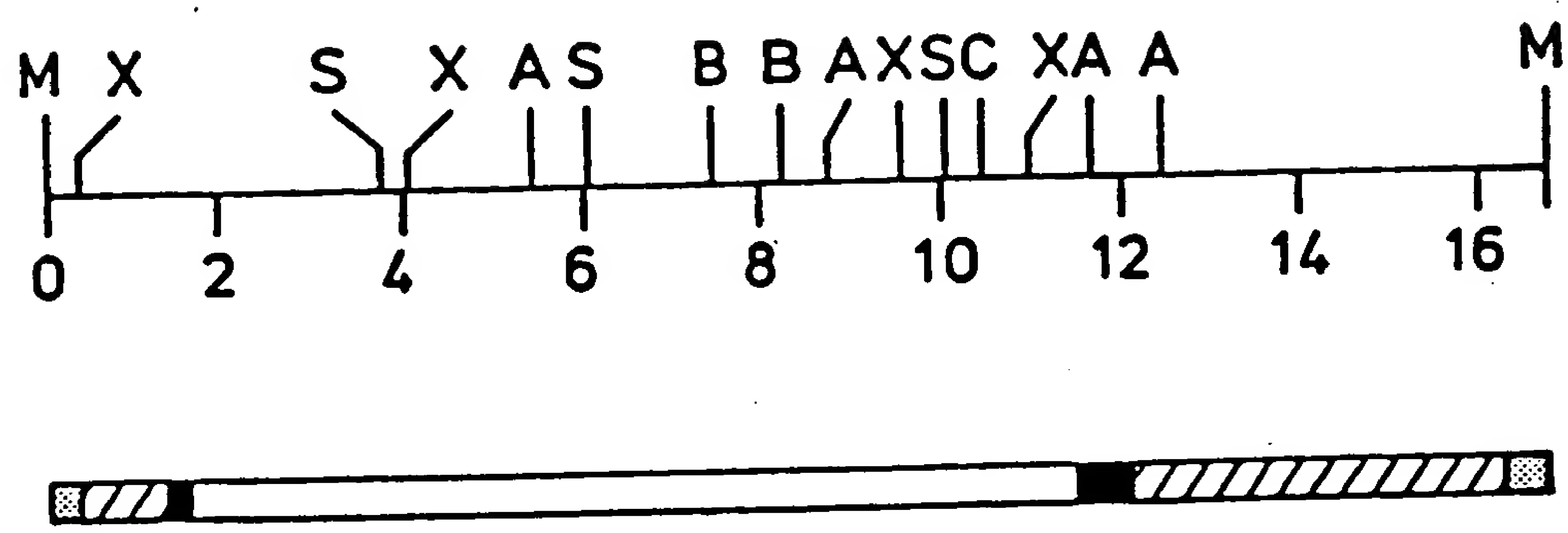
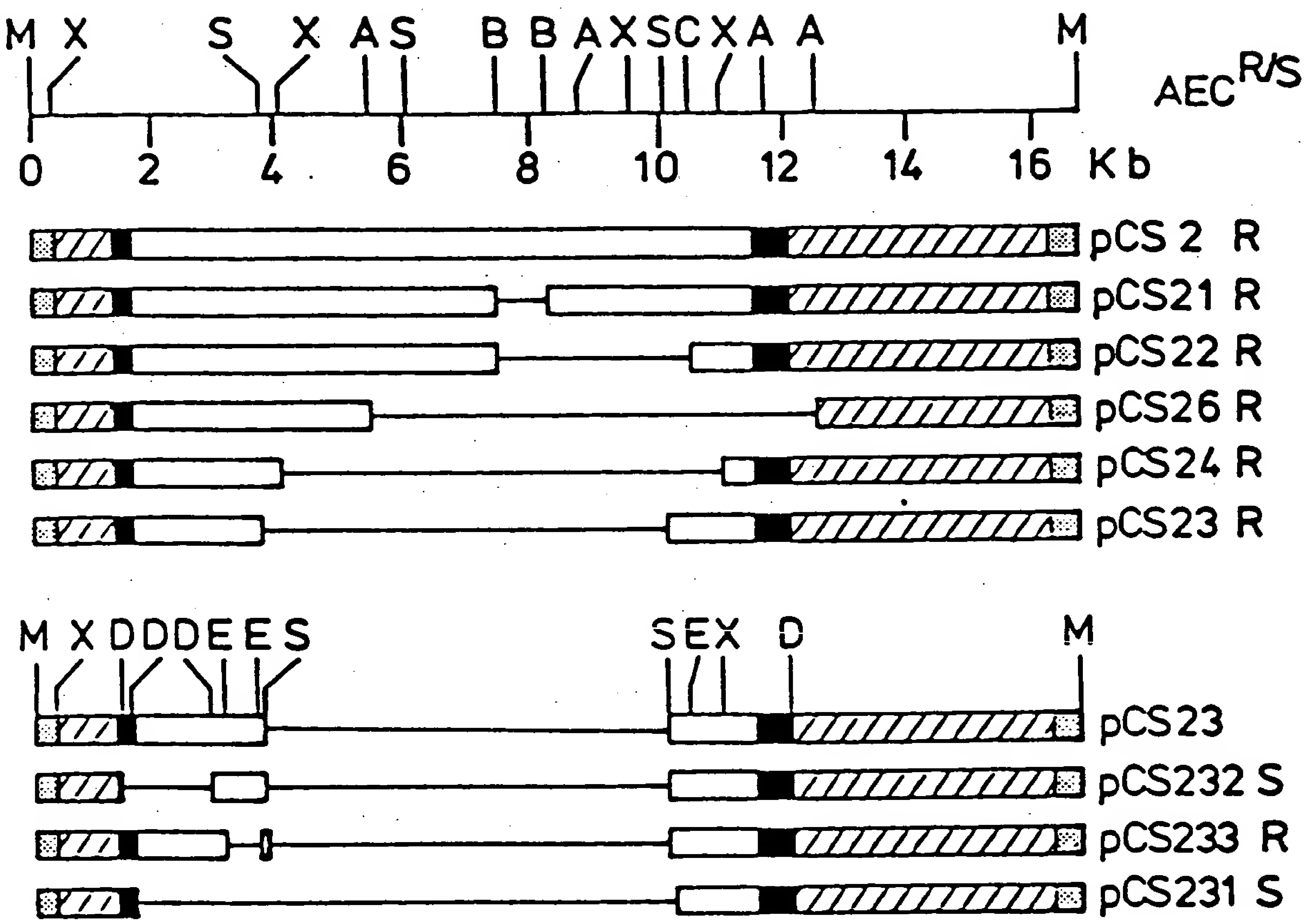


Figure 2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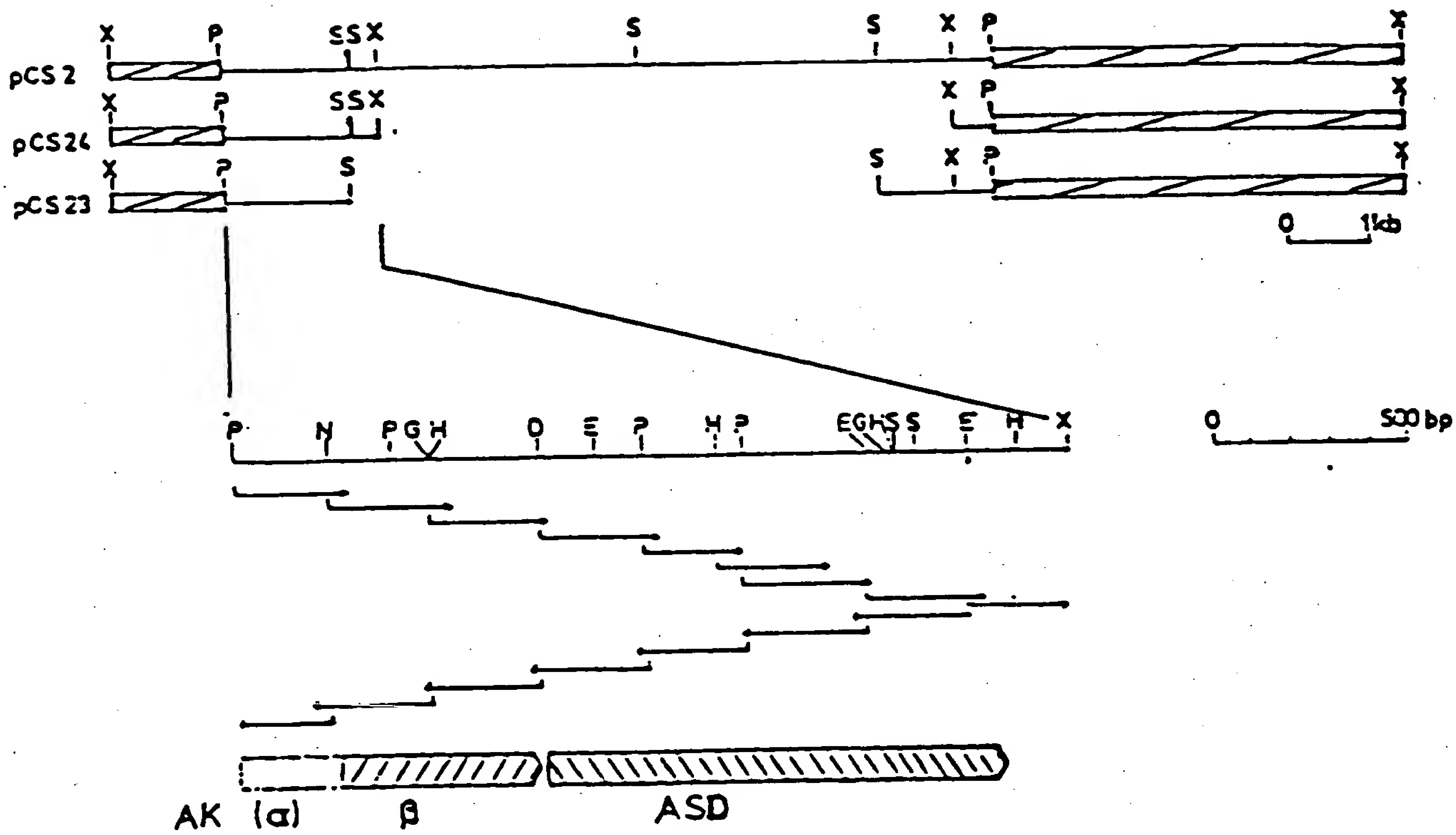
AEC R/S
0 2 4 6 8 10 12 14 16 Kb
pCS 2 R
pCS21 R
pCS22 R
pCS26 R
pCS24 R
pCS23 R
M X D D D E E S S E X D M
pCS23
pCS232 S
pCS233 R
pCS231 S

Figure 3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pCS 24
pCS 23
X
P
SSX
S
S
X
P
X
0
1kb
P
N
P G H
D
E
P
H P
EGHS S
E
H
X
0
500 bp
AK
(α)
β
ASD

Figure 4

```
 AlaValAlaLeuAlaAlaAlaLeuAsnAlaAspValCysGluIleTyrSerAspValAspGlyValTyrThrAlaAspProArgIleV
CTGCAGTTGCCGTTGGCAGCTGCTTTGAACGCTGATGTGTGTCAGATTTACTCGGACGTTGACGGTGTGTATACCGCTGACCCGCGCATCG
 PstI    10        20        30        40        50        60        70        80        90

 ProAsnAlaGlnLysLeuGluLysLeuSerPheGluGluMetLeuGluLeuAlaAlaValGlySerLysIleLeuValLeuArgSerV
TTCCTAATGCACAGAAGCTGGAAAAGCTCAGCTTCGAAGAAATGCTGGAACTTGCTGCTGTTGGCTCCAAGATTTTGGTGCTGCGCAGTG
        100       110       120       130       140       150       160       170       180

 GluTyrAlaArgAlaPheAsnValProLeuArgValArgSerSerTyrSerAsnAspProGlyThrLeuIleAlaGlySerMetGluA
TTGAATACGCTCGTGCATTCAATGTGCCACTTCGCGTACGCTCGTCTTATAGTAATGATCCCGGCACTTTGATTGCCGGCTCTATGGAGG
        190       200       210       220       230       240       250   NaeI        ***

 IleProValGluGluAlaValLeuThrGlyValAlaThrAspLysSerGluAlaLysValThrValLeuGlyIleSerAspLysProG
ATATTCCTGTGCAAGAAGCAGTCCTTACCGGTGTCGCAACCGACAAGTCCGAAGCCAAAGTAACCGTTCTGGGTATTTCCGATAAGCCAG
*       -->       290       300       310       320       330       340       350       360

 GluAlaAlaLysValPheArgAlaLeuAlaAspAlaGluIleAsnIleAspMetValLeuGlnAsnValTyrSerValGluAspGlyT
GCGAGGCTGCGAAGGTTTTCCGTGCGTTGGCTGATGCAGAAATCAACATTGACATGGTTCTGCAGAACGTCTATTCTGTAGAAGACGGCA
        370       380       390       400       410       420 PstI  430       440       450

 ThrAspIleThrPheThrCysProArgSerAspGlyArgArgAlaMetGluIleLeuLysLysLeuGlnValGlnGlyAsnTrpThrA
CCACCGACATCACCTTCACCTGCCCTCGTTCCGACGGCCGCCGCGCGATGGAGATCTTGAAGAAGCTTCAGGTTCAGGGCAACTGGACCA
        460       470       480       490       500 BglII 510  HindIII        530       540

 ValLeuTyrAspAspGlnValGlyLysValSerLeuValGlyAlaGlyMetLysSerHisProGlyValThrAlaGluPheMetGluA
ATGTGCTTTACGACGACCAGGTCGGCAAAGTCTCCCTCGTGGGTGCTGGCATGAAGTCTCACCCAGGTGTTACCGCAGAGTTCATGGAAG
        550       560       570       580       590       600       610       620       630

 LeuArgAspValAsnValAsnIleGluLeuIleSerThrSerGluIleArgIleSerValLeuIleArgGluAspAspLeuAspAlaA
CTCTGCGCGATGTCAACGTGAACATCGAATTGATTTCCACCTCTGAGATTCGTATTTCCGTGCTGATCCGTGAAGATGATCTGGATGCTG
        640       650       660       670       680       690       700       710       720

 AlaArgAlaLeuHisGluGlnPheGlnLeuGlyGlyGluAspGluAlaValValTyrAlaGlyThrGlyArgOC
CTGCACGTGCATTGCATGAGCAGTTCCAGCTGGGCGGCGAAGACGAAGCCGTCGTTTATGCAGGCACCGGACGCTAAAGTTTTAAAGGAG
        730       740       750       760       770       780       790       800 DraI****

          MetThrThrIleAlaValValGlyAlaProAlaArgSerAlaArgLeuCysAlaProPheTrpLysSerAlaIleSerGl
TAGTTTTACAATGACCACCATCGCAGTTGTTGGTGCACCGGCCAGGTCGGCCAGGTTATGCGCACCCTTTTGGAAGAGCGCAATTTCCCA
        820-->    830       840       850       860       870       880       890       900

 LeuThrLeuPheValSerLeuLeuProThrSerAlaGlyArgLysIleGluPheArgGlyThrGluIleGluValGluAspIleThrGl
GCTGACACTGTTCGTTTCTTTGCTCCCCACGTCCGCAGGCCGTAAGATTGAATTCCGTGGCACGGAAATCGAGGTAGAAGACATTACTCA
        910       920       930       940       950 EcoRI 960       970       980       990

 AlaThrGluGluSerLeuLysAspIleAspValAlaLeuPheSerAlaGlyGlyThrAlaSerLysGlnTyrAlaProLeuPheAlaAl
GGCAACCCAGGAGTCCCTCAAGGACATCGACGTTGCGTTGTTCTCCGCTGGAGGCACCGCTTCCAAGCAGTACGCTCCACTGTTCGCTGC
       1000      1010      1020      1030      1040      1050      1060      1070      PstI
```

Figure 5

89124 AM/BT

CUSHMAN FORM

FOR UTILITY DESIGN
CIP PLANT
ORIGINAL SUBSTITUTE
DECLARATIONS

RULE 63 (37 C.F.R. 1.63)

DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

As a below named inventor, I hereby declare that my residence, post office address and citizenship are as stated below next to my name, and I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

PROCESS FOR THE FERMENTATIVE PRODUCITION OF L-LYSINE

the specification of which (CHECK applicable BOX(ES))

☐ is attached hereto.

BOX(ES) X was filed on March 13, 1990 as U.S. Application No. 0

☐ was filed as PCT international Application No. PCT .... on ....

and (if applicable to U.S. or PCT application) was amended on ....

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims (as amended by any amendment referred to above) to the best of my ability. I acknowledge the duty to disclose information which is material to the examination of this application in accordance with 37 C.F.R. 1.56(a). I hereby claim foreign priority benefits under 35 U.S.C. 119/365 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate filed by me or my assignee disclosing the subject matter claimed in this application and having a filing date (1) before that of the application on which priority is claimed, or (2) if no priority claimed, before the filing date of this application:

PRIOR FOREIGN APPLICATION(S)

Number	Country	Day/MONTH/Year Filed	Priority Claimed Yes	Priority Claimed No
P39 08 201.6	West Germany	13/03/89	X	

I hereby claim the benefit under 35 U.S.C. 120/365 of all United States applications listed below and PCT international applications listed above or below and, insofar as the subject matter of each of the claims of this application is not disclosed in such prior applications in the manner provided by the first paragraph of 35 U.S.C. 112, I acknowledge the duty to disclose material information as defined in 37 C.F.R. 1.56(a) which occurred between the filing date of each such prior application and the national or PCT international filing date of this application:

PRIOR U.S. OR PCT APPLICATION(S)

Application No. (series code/serial no.)	Day/MONTH/Year Filed	Status pending, abandoned, patented

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

And I hereby appoint Cushman, Darby & Cushman, Eleventh Floor, 1615 L Street, N.W., Washington, D.C. 20036-5601, telephone number 861-3000 (to whom all communications are to be directed), and the below named partners thereof (of the same address) individually and collectively my attorneys to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith and with the resulting patent, and I hereby authorize them to act and rely on instructions from and communicate directly with the person/assignee/attorney/firm/organization who/which first sends/sent this case to them and by whom/which I hereby declare that I have consented after full disclosure to be represented unless/until I instruct Cushman, Darby & Cushman in writing to the contrary.

Paul N. Kokulis	16773	William K. West, Jr.	22057	William T. Bullinger	25503	Glenn J. Perry	28458
Raymond F. Lippitt	17519	Kevin E. Joyce	20508	Donald J. Bird	25323	Kendrew H. Colton	30368
G. Lloyd Knight	17698	Edward M. Prince	22429	W. Warren Taltavull	25647	Chris Comuntzis	31097
Carl G. Love	18781	Donald B. Deaver	23048	Watson T. Scott	26581	Lawrence Harbin	27644
Lawrence A. Hymo	19057	David W. Brinkman	20817	Peter W. Gowdey	25872	Walter G. Walker	27963
Edgar H. Martin	20534	George M. Sirilla	18221	Dale S. Lazar	28872	Paul E. White	32011

1) INVENTOR'S SIGNATURE Bernd Bachmann Date March 30 th 1990

Inventor's Name (typed) Bernd (First) (Middle Initial) BACHMANN (Family Name) W. German (Citizenship)

Residence (City) Werther (State/Foreign Country) West Germany

Post Office Address (Include Zip Code) Meyerfield 10a, D-4806 Werther, West Germany

2) INVENTOR'S SIGNATURE Georg Thierbach Date March 30 th 1990

Inventor's Name (typed) Georg (First) (Middle Initial) THIERBACH (Family Name) W. German (Citizenship)

Residence (City) Bielefeld (State/Foreign Country) West Germany

Post Office Address (Include Zip Code) Gunststrasse 21, D-4800 Bielefeld 1, West Germany

3) INVENTOR'S SIGNATURE Jörn Kalinowski Date April 2nd 1990

Inventor's Name (typed) Jörn (First) (Middle Initial) KALINOWSKI (Family Name) W. German (Citizenship)

Residence (City) Bielefeld (State/Foreign Country) West Germany

Post Office Address (Include Zip Code) Drögestrasse 25, D-4800 Bielefeld 1, West Germany

4) INVENTOR'S SIGNATURE Alfred Pühler Date April 2, 1990

Inventor's Name (typed) Alfred (First) (Middle Initial) PÜHLER (Family Name) W. German (Citizenship)

Residence (City) Bielefeld (State/Foreign Country) West Germany

Post Office Address (Include Zip Code) Am Waldschlösschen 2, D-4800 Bielefeld 15, West Germany

FOR ADDITIONAL INVENTORS, check box ☐ and attach sheet (CDC-116/2) for same information for each re signature, name, date, citizenship, residence and address.

CDC-116 Jan 91